METHODS AND COMPOSITIONS FOR IDENTIFYING VARIATIONS IN HUMAN α_{1B}- AND β_2-ADRENERGIC RECEPTOR GENES

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TECHNICAL FIELD

The present invention relates generally to the field of genetic analysis. More specifically, this invention relates to compositions and methods for identifying genetic alterations of the human α_{1B}- and β_2-adrenergic receptors (ARs). The compositions and methods involve α_{1B}- and β_2-AR specific primer sets that are particularly advantageous for their ability to amplify large gene segments suitable for automated DNA sequencing analysis. Generation of these particular primer sets facilitates large-scale rapid screening of genetic variations in these receptors, which are of major physiological and clinical significance.

BACKGROUND ART

Adrenergic receptors (ARs) constitute a multigene family of G-protein-coupled receptor proteins that mediate the physiological actions of important neurotransmitters and hormones, including in particular, epinephrine and norephinephrine. ARs are broadly divided into α and β types on the basis of their pharmacological specificity (R.P. Ahlquist, *Am. J. Physiol.* **153**:586-600, 1948); each class is further divided into several subtypes (e.g. α_{1A},α_{1B}, α_{1D}; α_{2A}, α_{2B}, α

$_{2C}$; and β_1, β_2, β_3) according to sequence homology, drug specificity, and mechanism of signal transduction. Within this heterogeneous family, the α_1-ARs play important roles in various physiological functions, including smooth muscle contraction, blood pressure homeostasis and cell growth control; whereas β_2-ARs play a role in regulation of cell metabolism and muscle relaxation (see e.g. P.A. Insel, *N. Engl. J. Med.* **334**:580-585, 1996; J.P. Raymond et al., *Hypertension* **15**:119-131, 1990). These subtypes of ARs are thus important targets for therapeutic agents, including drugs for cardiovascular diseases, asthma, prostatic hypertrophy and nasal congestion, among others.

As with other G-protein-coupled receptors, α_{1B}-AR and β_2-AR generally contain a characteristic group of seven transmembrane-spanning regions, an extracellular amino terminus with ligand binding sites, and an intracellular carboxyl terminus capable of interacting with heterotrimeric G-proteins to transduce downstream signals.

Human I_{1B}-AR gene is localized to region q31-q32 on chromosome 5. It consists of two exons and a single large intron of at least 20 kilobases, which interrupts the coding region at the end of the putative sixth transmembrane domain (C.S. Ramarao et al., *J. Biol. Chem.* **267**:21936-21945, 1992). The first exon encodes two thirds of the coding sequence, and the entire sequence predicts a polypeptide of 517 amino acids with a molecular weight of about 57 kDa. Alterations in I_{1B}-AR function have been associated with disorders, such as hypertension (M.C. Michel et al., *FASEB J.* **3**:139-144, 1989), prostatic hypertrophy (K. Shibata et al., *Br. Z. Pharmacol.* **118**:1403-1408, 1996) and malignant arrhythmia in myocardial ischemia (G.E.Billman et al., *J. Cardiovas. Pharmacol.* **24**:394-402, 1994). Of particular interest are mutations resulting in constitutively active I_{1B}-AR, characterized by their marked affinity to agonists, and their ability to signal through heterotrimeric G proteins in the absence of agonist. (R.D.Guarino et al., *Cell. Signal.* **8(5)**:323-333, 1996). Transgenic animals expressing altered ARs or altered levels of ARs provide a convenient means of testing for the effects of such alterations. For example, transgenic mice

expressing constitutively active I_{1B}-ARs in their thyroid cells developed hyperthyoridism and thyroid follicular cell malignancies (C. Ledent et al., *Endocrinology* **138(1)**:369-378, 1997); whereas transgenic mice deficient in I_{1B}-AR, exhibited decreased blood pressure response and attenuated vascular contraction when stimulated with I-agonists (A. Cavalli et al. P*roc. Natl. Acad. Sci.* USA, **94**: 11589-11594, 1997).

Human ϑ_2-AR is encoded by an intronless gene, mapped to the same chromosomal region in which I_{1B}-AR gene is located (J.R. Sheppard et al., *Proc. Natl. Acad.Sci. USA* **80**:233-236, 1983). The ϑ_2-AR locus is polymorphic. At least six polymorphic forms of ϑ_2-AR have been identified (S.B. Liggett et al., *Clinical and Experimental Allergy* **25(2)**:89-94, 1995). Exemplary polymorphisms consist of amino acid substitutions at position 16 (arginine to glycine), 27 (glutamine to glutamate), and 164 (threonine to isoleucine). Research over the past five years has strongly implicated a causal role of ϑ_2-AR polymorphism in the pathogenesis of asthma. Specifically, certain polymorphisms appear to contribute to baseline hyper-reactivity, play a role in the severity of asthma, and dictate certain asthmatic phenotypes. By way of illustration, glycine 16 polymorphism has been associated with nocturnal asthma (J. Turki et al., *J. Clin. Invest.* **95**:1635-1641, 1995). The substitution of arginine with glycine apparently imparts an enhanced downregulation of the receptor, which is consistent with the nocturnal downregulation of ϑ_2-AR and the diminished response to ϑ-agonists observed in nocturnal asthma. The same polymorphic variant was noted in essential hypertension in African Caribbeans (P. Kontanko et al., *Hypertension* **30(4)**: 773-776, 1997).

Traditionally, the identification of genetic alterations, particularly mutations and polymorphisms that are associated with disease states, has typically been proceeded by two approaches; neither of which is generally amenable for large-scale diagnostic screens or suitable for all genetic analyses. The first approach involves direct analysis of the target gene, employing techniques such as (a) direct hybridization of a gene-specific probe; (b) the use of a mutation-specific

restriction endonuclease; and (c) the use of oligonucleotide probes to detect a point mutation. Gene-specific probes are generally limited to detection of a recessive genetic disorder resulting from a deletion of part or all of the genomic DNA sequence complementary to the DNA probe. If the entire probe sequence is deleted, the homozygous condition is characterized by the complete absence of any DNA fragments that can be recognized by the probe. The second method, employing a mutation-specific restriction endonuclease, is only useful when the molecular defect responsible for the disorder abolishes or creates a new restriction enzyme recognition site. Thirdly, the use of oligonucleotide probes is generally restricted to detection of genetic alterations in which the exact sequences of the normal and mutant gene are known. To cover a spectrum of mutations causing the same disease phenotype, a battery of oligonucleotide probes is thus required. Therefore, oligonucleotide probes are not desirable for disorders that arise from previously unrecognized new mutations.

The second approach for identifying genetic alterations involves an indirect analysis by restriction fragment length polymorphism (RFLP). RFLP occurs if a variation in DNA sequence creates a new restriction enzyme site or abolishes an existing one. There are many technical disadvantages affecting the use of RFLPs. The major problem is the inability of a test to detect changes that do not affect cleavage with a restriction endonuclease. The other shortcoming is the potential for recombination between the disease gene and the polymorphic site, which render a pedigree analysis of genetic diseases uninformative. To be useful for diagnosis, a polymorphic marker must be closely linked to the site of the disease mutation. However, even when the RFLPs are only a few kilobase pairs away from the disease gene, the chance of recombination may still be quite high if a hotspot for DNA recombination is located between the markers and the mutation. Moreover, as in many of the test methods aforementioned, detection of RFLPs is expensive, labor intensive, and time consuming.

For many disorders, the location and nature of the molecular defects are unknown. Among genes that have been investigated for structural aberrations

leading to human genetic diseases, most of the sequence variants involve changes in single nucleotide positions. This is also the case for α_{1B}-AR and β_2-AR variants identified thus far. Therefore, it appears that an ideal method for screening genetic alterations associated with clinical states should allow (a) a rapid amplification of the target gene from a complex mixture of nucleic acids; and (b) direct DNA sequencing of the resulting amplified products to determine every existing single base change.

Polymerase chain reaction (PCR) and automated nucleotide sequencing techniques can meet these criteria and have successfully been employed for the identification of abnormalities in some but not all disease genes. It is a well-known problem in the art that standard protocols for PCR are not applicable for every new application. For instance, attempts to isolate the entire coding sequence of ϑ_2-AR or I_{1B}-AR gene (or even its two individual exons) from a complex mixture of genomic DNA by PCR for a sequence analysis have not until now been reported. Some often-encountered problems include lack of detectable product or a low yield of the desired product; heterogeneity of products due to mispriming or misextension; and the formation of "primer-dimers" that compete for amplification with the desired product. To increase the frequency of the desired products, further genetic manipulations such as subcloning a mixture of PCR products and screening for the desired DNA fragment are required prior to nucleotide sequencing. However, these procedures render automation of the DNA analysis of α_{1B}-AR and ϑ_2-AR genes much more difficult and infeasible on a large scale.

There thus remains a considerable need for compositions and methods that allow for a rapid isolation of α_{1B}-AR and ϑ_2-AR genes in large quantity and substantial homogeneity, which is necessary for automated sequencing analysis. The development of these compositions and methods would greatly facilitate screening for genetic abnormalities of I_{1B}-AR and ϑ_2-AR that are associated with disease processes.

SUMMARY OF THE INVENTION

The present invention provides compositions that can be used to generate a fast and specific amplification of the coding sequences of the human I_{1B}-AR and ϑ_2-AR genes. Specifically, the invention provides I_{1B}-AR- and ϑ_2-AR-specific primers capable of generating large DNA segments in substantial homogeneity. The invention also provides a method of using these primers for amplifying I_{1B}-AR and ϑ_2-AR gene segments, a method of using the resultant products for the identification of genetic alterations in these receptors, and a method of diagnosing diseases associated with the detected alterations in these receptor genes.

Accordingly, one embodiment of this invention is a primer pair for amplifying an I_{1B}-AR gene which exhibits preferably a majority of the following characteristics, more preferably most of the listed characteristics, even more preferably all of the following characteristics: a) each individual primer of a pair is non-self hybridizing, contains at least 15 nucleotides, and has a melting temperature within the range of 50°C to 85°C; b) the selected pair is non-cross hybridizing; c) the selected pair anneals to two distinct regions, which are separated by a distance of at least about 400 nucleotides within the gene; d) the selected pair produces a substantially homogenous plurality of amplified fragments in a polymerase chain reaction; and e) at least one primer of the pair is capable of extending its 3' end sequence complementary to the template sequence in a DNA polymerase reaction.

In one aspect of this embodiment, an I_{1B}-AR gene specific primer pair amplifies a fragment of exon 1 selected from the group consisting of the N-terminal region A and the C-terminal region B as shown in Figure 1. Fragment A can be amplified by a primer pair having two primers each comprising a linear sequence, preferably essentially identical to the polynucleotide shown in SEQ ID NO:1 or 2; more preferably, it is amplified by two primers each having a linear sequence identical to the polynucleotide shown in SEQ ID NO:1 or 2. Fragment B can be amplified by a primer pair having two primers each comprising a linear sequence, preferably essentially identical to the polynucleotide shown in SEQ ID

NO:3 or 4; more preferably, it is amplified by two primers each having a linear sequence identical to the polynucleotide shown in SEQ ID NO:3 or 4.

A further embodiment of this invention is a primer pair for amplifying ϑ_2-AR gene, having preferably a majority of the following characteristics, more preferably most of the characteristics, even more preferably all of the following characteristics: a) each individual primer of a pair is non-self hybridizing, contains at least 15 nucleotides, and has a melting temperature within the range of 50°C to 85°C; b) the selected pair is non-cross hybridizing; c) the selected pair anneals to two distinct regions, which are separated by a distance of at least about 400 nucleotides within the gene; d) the selected pair produces a substantially homogenous plurality of amplified fragments in a polymerase chain reaction; and e) at least one primer of the pair is capable of extending its 3' end sequence complementary to the template sequence in a DNA polymerase reaction. In one aspect of this embodiment, a ϑ_2-AR gene specific primer pair amplifies a fragment of the coding region selected from the group consisting of the N-terminal region A and the C-terminal region B as shown in Figure 2. Preferably, fragment A is amplified by a primer pair having two primers each comprising a linear sequence, preferably essentially identical to the polynucleotide shown in SEQ ID NO:5 or 6; more preferably, it is amplified by two primers each having a linear sequence identical to the polynucleotide shown in SEQ ID NO:5 or 6. Fragment B is amplified by a primer pair having two primers each comprising a linear sequence, preferably essentially identical to the polynucleotide shown in SEQ ID NO:7 or 8; more preferably, it is amplified by two primers each having a linear sequence identical to the polynucleotide shown in SEQ ID NO:7 or 8.

Another embodiment of this invention is a method of amplifying a segment of the coding sequence of I_{1B}-AR gene of a subject. Still another embodiment of this invention is a method of amplifying a segment of the coding sequence of ϑ_2-AR gene of a subject. Each method generally comprises the steps of providing a biological sample containing nucleic acid molecules from the subject; employing a pair of I_{1B}-AR or ϑ_2-AR gene specific primers as described

herein, a primer-dependent DNA polymerase, and a sufficient amount of deoxyribonucleotides to generate a plurality of gene segments.

Another embodiment of this invention is a method of identifying a genetic variation in I_{1B}-adrenergic receptor gene of a subject. Still another embodiment of this invention is a method of identifying a genetic variation in ϑ_2-AR gene. Each method generally comprises the steps of providing a biological sample containing nucleic acid molecules from the subject; amplifying a segment of the gene by employing at least one of the primer pairs of the present invention as described herein; and identifying sequence variations of the resultant products relative to a control involving one or more of the following analytical steps, including nucleotide sequencing, single-strand conformation polymorphism assay, allele-specific oligonucleotide hybridization, Southern blot analysis, and restriction endonuclease digestion.

Yet another embodiment of this invention is a method for diagnosing diseases associated with genetic alterations of I_{1B}-adrenergic receptor. A further embodiment of this invention is a method for diagnosing diseases based on genetic alterations of β_2-adrenergic receptor. Each method generally comprises the steps of providing a biological sample of the subject containing nucleic acid molecules; amplifying a segment of the gene by employing at least one of the primer pairs of the present invention as described herein, a primer-dependent DNA polymerase, and a sufficient amount of deoxyribonucleotides to generate a plurality of amplified segments of the gene; screening the resultant segments for genetic variations relative to a control involving at least one analytical step as listed above; and determining a correlation between the detected variations in the subject and a control. Diseases resulting from alterations in I_{1B}-AR and β_2-AR can include cardiovascular, peripheral vascular, pulmonary, prostatic and neuro-psychic, and endocrine-metabolic disorders.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 (SEQ ID NO:9) depicts the DNA sequence encoding exon 1 of the human α_{1B}-adrenergic receptor gene. Nucleotide numbering starts with 1 of the noncoding sequence as published (C.S. Ramarao et al., *J. Biol. Chem.* **267**:21936-21945). The coding region is shown in upper case letters. Intron and noncoding sequences are shown in lower case letters. Only a partial intron sequence is shown. Sites for potential N-linked glycosylation are indicated with triangles. Exemplary primers (SEQ ID NOS: 1-4) are shown as boxed in Figure 1. The sequence segment amplified by primers SEQ ID NOS:1 and 2 is denoted A, and that amplified by primers SEQ ID NOS:3 and 4 is denoted B.

Figure 2 (SEQ ID NO:10) depicts the DNA sequence encoding the human ϑ_2-adrenergic receptor gene. Nucleotide numbering starts with 1 of the noncoding sequence as published (B.K. Kobilka et al. *Proc. Natl. Acad. Sci. USA* **84**:46-50, 1987). The coding region is shown in upper case letters. The noncoding sequence is shown in lower case letters. Exemplary primers (SEQ ID NOS: 5-8) are shown as boxed in Figure 2. The sequence segment amplified by primers SEQ ID NOS:5 and 6 is denoted A, and that amplified by primers SEQ ID NOS:7 and 8 is denoted B.

Figure 3 depicts a schematic representation of the genomic organization of the human I_{1B}- AR gene. Panel A is a restriction map of the gene. The line representing the gene is interrupted at the location corresponding to the gap in the intron. Exons are indicated by solid boxes. Restriction sites are indicated by vertical lines. Panel B details the restriction sites of the coding region: B, BamHI; E, EcoRI; K, KpnI; N, NotI; P, PstI; S, SmaI; X, XhoI. Putative cyclic AMP responsive element and polyadenylation sites are indicated.

Figure 4 is a representation of an agarose gel electrophoretic separation of amplified PCR products. Human I_{1B}-AR gene segments A and B (see Figure 1) are shown in lanes 1 and 2. Human ϑ_2-AR gene segments A and B (see Figure 2) are shown in lanes 3 and 4, respectively. DNA molecular weight standards are included to indicate the electrophoretic mobility of the amplified products.

Figure 5 depicts the sequence profile of fragment A of the human α_{1B}-AR gene. The amplified fragments were sequenced with the amplification primer SEQ ID NO:1 using Applied Biosystems' DNA sequencer. Each peak indicates a different nucleotide. In some cases "ambiguous reads" (N) occur as the result of duplicate bases (heterozygosity) or reading ambiguities, which can generally be corrected afterwards.

Figure 6 depicts the sequence profile of fragment A of the human β_2-AR gene. The amplified fragments were sequenced with the amplification primer SEQ ID NO:6 using Applied Biosystems' DNA sequencer. Each peak indicates a different nucleotide. In some cases "ambiguous reads" (N) occur as the result of duplicate bases (heterozygosity) or reading ambiguities, which can generally be corrected afterwards.

DETAILED DESCRIPTION OF THE INVENTION

As noted above, this invention provides compositions and methods for a rapid amplification of the coding sequence of a α_{1B}-AR gene or a ϑ_2-AR gene, to generate a population of substantially homogenous gene segments. The amplified gene segments can be directly used for a high throughput screen of sequence variants, e.g., by automated DNA sequencing analysis. Accordingly, the invention provides a method of identifying genetic alterations of α_{1B}-AR or ϑ_2-AR by detecting changes in the gene nucleotide sequences. The invention further provides a method for diagnosing diseases resulting from genetic alterations of these receptors.

Definitions

The term "polynucleotide" or "nucleic acid" refer to a polymeric form of nucleotides of any length, either deoxyribonucleotides or ribonucleotides, or

analogs thereof. The terms "polynucleotide" and "nucleotide" as used herein are used interchangeably. Polynucleotides may have any three-dimensional structure, and may perform any function, known or unknown. The following are non-limiting examples of polynucleotides: a gene or gene fragment, exons, introns, messenger RNA (mRNA), transfer RNA, ribosomal RNA, ribozymes, cDNA, recombinant polynucleotides, branched polynucleotides, plasmids, vectors, isolated DNA of any sequence, isolated RNA of any sequence, nucleic acid probes, and primers. A polynucleotide may comprise modified nucleotides, such as methylated nucleotides and nucleotide analogs. If present, modifications to the nucleotide structure may be imparted before or after assembly of the polymer. The sequence of nucleotides may be interrupted by non-nucleotide components. A polynucleotide may be further modified after polymerization, such as by conjugation with a labeling component. A "fragment" or "segment" of a nucleic acid is a small piece of that nucleic acid.

A "gene" refers to a polynucleotide containing at least one open reading frame that is capable of encoding a particular protein after being transcribed and translated.

A "primer" is a short polynucleotide, generally with a free 3' -OH group, that binds to a target or "template" potentially present in a sample of interest by hybridizing with the target, and thereafter promoting polymerization of a polynucleotide complementary to the target. A "polymerase chain reaction" ("PCR") is a reaction in which replicate copies are made of a target polynucleotide using a "pair of primers" or a "set of primers" consisting of an "upstream" and a "downstream" primer, and a catalyst of polymerization, such as a DNA polymerase, and typically a thermally-stable polymerase enzyme. Methods for PCR are well known in the art, and taught, for example in "PCR: a practical approach" (M. MacPherson et al., IRL Press at Oxford University Press 1991). All processes of producing replicate copies of a polynucleotide, such as PCR or gene cloning, are collectively referred to herein as "replication."

In the context of polynucleotides, a "linear sequence" or a "sequence" is an order of nucleotides in a polynucleotide in a 5' to 3' direction in which residues that neighbor each other in the sequence are contiguous in the primary structure of the polynucleotide.

"Hybridization" refers to a reaction in which one or more polynucleotides react to form a complex that is stabilized via hydrogen bonding between the bases of the nucleotide residues. The hydrogen bonding may occur by Watson-Crick base pairing, Hoogstein binding, or in any other sequence-specific manner. The complex may comprise two strands forming a duplex structure, three or more strands forming a multi-stranded complex, a single self-hybridizing strand, or any combination of these. A hybridization reaction may constitute a step in a more extensive process, such as the initiation of a PCR reaction, or the enzymatic cleavage of a polynucleotide by a ribozyme.

Hybridization reactions can be performed under conditions of different "stringency". Relevant conditions include temperature, ionic strength, time of incubation, the presence of additional solutes in the reaction mixture such as formamide, and the washing procedure. Higher stringency conditions are those conditions, such as higher temperature and lower sodium ion concentration, which require higher minimum complementarity between hybridizing elements for a stable hybridization complex to form. Conditions that increase the stringency of a hybridization reaction are widely known and published in the art: see, for example, "Molecular Cloning: A Laboratory Manual", Second Edition (Sambrook, Fritsch & Maniatis, 1989).

When hybridization occurs in an antiparallel configuration between two single-stranded polynucleotides, the reaction is called "annealing" and those polynucleotides are described as "complementary". A double-stranded polynucleotide can be "complementary" or "homologous" to another polynucleotide, if hybridization can occur between one of the strands of the first polynucleotide and the second. "Complementarity" or "homology" (the degree that one polynucleotide is complementary with another) is quantifiable in terms of

the proportion of bases in opposing strands that are expected to form hydrogen bonding with each other, according to generally accepted base-pairing rules.

"Melting temperature" of a primer refers to the temperature at which 50% of the primer-template duplexes are dissociated. Melting temperature is a function of ionic strength, base composition, and the length of the primer. It can be empirically determined, e.g., by measuring shift in optical density or can also be predicted using either of the following equations:

$$T_m\ (°C) = 81.5 + 16.6 \times \log [Na] + 0.41 \times (\%GC) - 600/N$$

where [Na] is the concentration of sodium ions, and the % GC is in number percent, where N is chain length, or

$$T_m\ (°C) = 2 \times (A+T) + 4 \times (C+G)$$

where A, T, G and C represent the number of adenosine, thymidine, guanosine and cytosine residues in the primer.

"Denaturation" refers to the process by which a double-stranded nucleic acid is converted into its constituent single strands. Denaturation can be achieved, for example, by the use of high temperature, low ionic strength, acidic or alkaline pH, and/or certain organic solvents. Methods for denaturing nucleic acids are well known in the art.

"Non-self hybridizing" as applied to a primer, means that the primer is essentially incapable of forming intra-molecular duplex mediated by hydrogen-bonding between the bases of the nucleotide residues within the primer under hybridization conditions of high stringency. A "non-self hybridizing" primer is essentially free of intra-molecular duplex preferably under a high stringency condition, more preferably under a moderately stringent condition, even more preferably under a low stringent condition. Factors determining hybridization stringency are well known in the art, and exemplary conditions of different stringency are provided in the descriptions that follow. The term "non-cross hybridizing" as applied to primer pairs means that the two individual primers do not hybridize and form a duplex stabilized by hydrogen-bonding between

complementary bases in the two primers under hybridization conditions of high stringency.

In determining whether a primer is non-self hybridizing or a primer pair is non-cross hybridizing, sequence complementarity within the primer and between the primers should be examined. A non-self hybridizing primer is one that lacks internal sequence homology necessary for duplex formation. Similarly, a pair of non-cross hybridizing primers does not share a sufficient amount of sequence homology to form a stable double-stranded structure.

A linear sequence of nucleotides is "identical" to another linear sequence, if the order of nucleotides in each sequence is the same, and occurs without substitution, deletion, or material substitution. It is understood that purine and pyrimidine nitrogenous bases with similar structures can be functionally equivalent in terms of Watson-Crick base-pairing; and the inter-substitution of like nitrogenous bases, particularly uracil and thymine, or the modification of nitrogenous bases, such as by methylation, does not constitute a material substitution. An RNA and a DNA polynucleotide have identical sequences when the sequence for the RNA reflects the order of nitrogenous bases in the polyribonucleotides, the sequence for the DNA reflects the order of nitrogenous bases in the polydeoxyribonucleotides, and the two sequences satisfy the other requirements of this definition. Where one or both of the polynucleotides being compared is double-stranded, the sequences are identical if one strand of the first polynucleotide is identical with one strand of the second polynucleotide.

A linear sequence of nucleotides is "essentially identical" to another linear sequence, if both sequences are capable of hybridizing to form a duplex with the same complementary polynucleotide. Sequences that hybridize under conditions of greater stringency are more preferred. It is understood that hybridization reactions can accommodate insertions, deletions, and substitutions in the nucleotide sequence. Thus, linear sequences of nucleotides can be essentially identical even if some of the nucleotide residues do not precisely correspond or align. In general, essentially identical sequences of about 40 nucleotides in length

will hybridize at about 40°C in 10X SSC (low stringency) or solution with equivalent ionic strength / temperature; preferably, they will hybridize at about 40 °C in 6 x SSC; more preferably, they will hybridize at about 50 °C in 6 x SSC (moderate stringency); even more preferably, they will hybridize at about 60 °C in 6 x SSC, or at about 40 °C in 0.5 x SSC, or at about 30 °C in 6 x SSC containing 50% formamide; still more preferably, they will hybridize at 40 °C or higher in 2 x SSC or lower in the presence of 50% or more formamide or equivalent ionic strength / temperature (high stringency). It is understood that the rigor of the test is partly a function of the length of the polynucleotide; hence shorter polynucleotides with the same homology should be tested under lower stringency and longer polynucleotides should be tested under higher stringency, adjusting the conditions accordingly. The relationship between hybridization stringency, degree of sequence identity, and polynucleotide length is known in the art and can be calculated by standard formulae. Sequences that correspond or align more closely to the invention disclosed herein are comparably more preferred. Generally, essentially identical sequences are at least about 80% identical with each other, after alignment of the homologous regions. Preferably, the sequences are at least about 85% identical; more preferably, they are at least about 90% identical; more preferably, they are at least about 95% identical; still more preferably, the sequences are 100% identical.

In determining whether nucleic acid sequences are essentially identical, a sequence that preserves the functionality of the nucleic acid with which it is being compared is particularly preferred. Functionality may be established by different criteria, such as ability to hybridize with a target polynucleotide, ability to effectively amplify a target sequence to yield a substantially homogenous multiplicity of products, and the ability to extend the 3' end sequence complementary to a target sequence in a DNA polymerase reaction, such as a nucleotide sequencing reaction. Primers having linear sequences that are essentially identical to those of the exemplary primers are considered as "derivatives".

As used herein, a "substantially homogenous plurality" of amplified products refers to a mixture of DNA fragments in which the undesired DNA fragments as indicated by, e.g., unexpected molecular sizes, constitute less than about 25% of the total amount of products. Preferably, the undesired fragments constitute less then 20%, more preferably 15% and even more preferably 10%. Product homogeneity may be indicated by a number of means, such as agarose gel electrophoresis of the amplified products, followed by visualizing a single DNA band upon staining the gel. Product homogeneity may also be determined by quantitative Southern blot analysis using the same primer pair employed in the amplification as probe. A ratio of 75% or above between the intensities of the band of predicted size and the bands of unexpected sizes indicates that the amplified products are substantially homogenous. Preferably, the ratio is above 80%, more preferably it is above 85%, even more preferably it is above 90%, still more preferably it is above 95%.

A "subject" or an "individual" refers to a human being.

A "genetic alteration" or "genetic variation" refers to a mutation or polymorphism in the nucleotide sequence of a given polynucleotide that may result in subsequent changes in the amino acid sequence of the encoded polypeptides. Mutations may arise from insertion, deletion, substitution or translocation of one or more nucleotide residues. A subject carries an altered or variant receptor gene when a difference in nucleotide sequence relative to that of a control is observed. A "control" is an alternative subject or sample used in an experiment for comparison purpose. A control can be "positive" or "negative". For example, where the purpose of the experiment is to determine a correlation of certain genetic alterations with a particular disease, it is generally preferable to use a positive control (a subject or a sample from a subject, carrying such a genetic alteration and exhibiting syndromes characteristic of that disease), and a negative control (a subject or a sample from a subject lacking the genetic alteration and clinical syndrome of that disease).

Selection of α_{1B}-AR or ϑ_2-AR gene specific primers

A central aspect of the invention is the design of DNA primer pairs capable of amplifying large fragments of I_{1B}- or ϑ_2-AR coding sequences from a complex mixture of nucleic acids to yield a substantially homogenous population of products that can be directly analyzed by automated nucleotide sequencing. The primer pairs of the present invention exhibit preferably a majority of the following characteristics, more preferably most of the characteristics, and even more preferably all of the characteristics as listed below:

a) each individual primer of a pair is non-self hybridizing, contains at least 15 nucleotides, and has a melting temperature within the range of 50°C to 85°C;

b) the selected pair is non-cross hybridizing;

c) the selected pair anneals to two distinct regions of the receptor gene, and said regions are separated by a distance of at least about 400 nucleotides;

d) the selected pair produces a substantially homogenous plurality of amplified fragments in a polymerase chain reaction; and

e) at least one primer of the pair is capable of extending its 3' end sequence complementary to the template sequence in a DNA polymerase reaction.

Several factors apply to the design of primer pairs having the above-mentioned characteristics. Firstly, the selected primer pair should contain sequences that are specific to the receptor genes and unique to the entire genome. Secondly, the two opposing primers of a pair should have similar thermal profiles and internal stability. This can be achieved by selecting primers with comparable length and G/C content. Preferred primers are from 18 to 23 nucleotide in length, having 50 to 60% G+C composition. In addition, the two opposing primers should not contain sequences that share internal homology or homology to each other. Extensive homology within the primer, e.g. due to inverted repeats,

promotes self-hybridization; complementarity of the two primers to each other, especially at their 3' ends, can cause cross-hybridization of primers and the generation of primer-dimers.

In a preferred embodiment, a primer pair generates a large fragment of the I_{1B}-AR gene, encompassing either the N-terminal half of the coding sequences of exon 1 as shown in region A of Figure 1, or the C-terminal half of the coding sequences as shown in region B of Figure 1. Preferred primer pairs replicate fragments of at least about 400 nucleotides within region A or B. Applying these criteria, by way of illustration, an exemplary primer pair that amplifies the N-terminal fragment of the I_{1B}-AR geneincludes an upstream and a downstream primer, having the nucleotide sequence 5'-CGGGGGAAGCAAAGTTTCA-3' (SEQ ID NO:1) and 5'-CGGCAGTACATGACTAGAAT-3' (SEQ ID NO:2), respectively. Another exemplary pair of primers that amplifies the C-terminal fragment consists of two opposing primers having the nucleotide sequence 5'-CTCTCCTTGGGTGGA AGGA-3' (SEQ ID NO:3) and 5'-AGCTCATCAGTAAACCCAAG-3' (SEQ ID NO:4), respectively. The resulting N-terminal fragment contains 766 nucleotide bases, and the C-terminal fragment has 473 nucleotide bases. Also encompassed by the invention are primers essentially identical to the two aforementioned primer sets which are selected to meet the criteria described above. An essentially identical primer set retains the characteristics and functionalities of the two exemplified primer pairs, even if they may contain mismatched nucleotide sequences. It is known in the art that a "perfectly matched" primer is not needed for a specific amplification. Minor changes in primer sequence achieved by substitution, deletion or insertion of a small number of bases do not affect the amplification specificity. For instance, primers may contain 5' extensions or mismatches for incorporating restriction enzyme sites, an ATG start codon, or promoter sequences into the target sequence. Mismatched bases can also be placed internally. In general, as much as 20% base-pair mismatch (when optimally aligned) can be tolerated. Changes in nucleotide residues positioned at

the 5' end of a primer is preferred over changes of that at the 3'end. Alteration of nucleotides located in the 3' end is preferred over changes in the middle of the primer.

In another preferred embodiment, a primer pair generates a large fragment of β_2-AR gene, encompassing either the N-terminal half of the coding sequences as shown in region A of Figure 2, or the C-terminal half of the coding sequences as shown in region B of Figure 2. Preferred primer pairs replicate fragments of at least about 400 nucleotides within region A or B. An exemplary primer pair that amplifies the N-terminal fragment includes an upstream and a downstream primer, having the nucleotide sequence 5'-GAATGAGGCTTCCAGGCGTC-3' (SEQ ID NO:5) and 5'-GATGATGCCTAACGTCTTG-3' (SEQ ID NO:6), respectively. The other exmeplary pair of primers that amplifies the C-terminal fragment consists of two opposing primers having the nucleotide sequence 5'-TTCTACGTGCCCCTG GTG-3' (SEQ ID NO:7) and 5'-TCCTCTAGGACTAAAGCTC-3' (SEQ ID NO:8), respectively. The resulting N-terminal fragment contains 917 nucleotide bases, and the C-terminal fragment has approximately 862 nucleotide bases. Also encompassed by the invention are primers essentially identical to the above-described two primer pairs.

Primers of the present invention can contain modified nucleotides, such as those conjugated with fluorophores, radioactive labels, and various chemical couplers including but not limited to biotin, alkaline phosphatase, horseradish peroxidase and digoxigenin. Primers provided by the invention may be either phosphorylated or non-phosphorylated at their 5'ends. It is within the skill of the art to prepare either type of primer by automated synthesis (see, e.g., Applied Biosystems Model 380D DNA synthesizer User's Manual). Phosphorylated oligonucleotides are synthesized on an automated instrument by using a phosphorylated monomer in the final coupling cycle. Such a phosphorylated monomer for use in automated synthesis may be obtained commercially, for example from Clontech Laboratories, Palo Alto, CA or Applied Biosystems, Foster City, CA or Glen Research, Sterling, VA or other commercial vendors.

Alternatively, a phosphorylated oligonucleotide may be prepared through automated synthesis of a 5'-hydroxyl-terminated oligonucleotide, followed by enzymatic phosphorylation using methods well-known in the art (e.g., Maniatis et al., Sambrook et al., Ausubel et al., supra). Preferably, synthesized primers are purified prior to gene amplification.

Uses of the human I_{1B}-AR and ϑ_2-AR gene specific primer sets

Use of the primer sets provided by this invention provides rapid amplification of the coding sequences of I_{1B}-AR or ϑ_2-AR gene to yield discrete gene segments in large quantity and substantial homogeneity. The amplified products are suitable for direct sequence analysis for identifying variations in nucleotide sequences, and therefore for diagnosing genetic diseases based on the altered sequences. The amplified products can also be used for generating receptor proteins for structure determination, to assay a molecule's activity on these receptors, and to screen for receptor-interacting molecules useful as diagnostics and/or therapeutics.

Amplification of the human I_1B-AR and ϑ_2-AR genes

In one embodiment, this invention provides a method for amplifying the coding sequence of exon 1 in the I_{1B}-AR gene of a subject. In another embodiment, the invention provides a method of amplifying the entire coding sequence of ϑ_2-AR gene. Each method generally comprises the following steps: a) providing a biological sample containing nucleic acid molecules from the subject; b) employing a primer pair as described herein, a primer-dependent polymerase, and a sufficient amount of deoxyribonucleotides to generate a plurality of gene segments in a polymerase reaction.

Biological samples used for this invention encompass body fluid, solid tissue samples, tissue cultures or cells derived therefrom and the progeny thereof, and sections or smears prepared from any of these sources, or any other samples expected to contain genetic material of the subject. Nucleic acid contained in

these samples can be extracted according to standard methods in the art, e.g., as illustrated herein (Example 3). For instance, DNA and RNA can be isolated using various lytic enzymes or chemical solutions according to the procedures set forth in Sambrook et al. ("Molecular Cloning: A Laboratory Manual", Second Edition,1989), or extracted by nucleic-acid-binding resins following the accompanying instructions provided by manufactures.

For the purpose of this invention, amplification means any method employing a primer-dependent polymerase capable of replicating a target sequence with reasonable fidelity. Amplification may be carried out by natural or recombinant DNA-polymerases such as T7 DNA polymerase, Klenow fragment of E. coli DNA polymerase, and reverse transcriptase.

A preferred amplification method is PCR. General procedures for PCR are taught in "PCR: a practical approach" (M. MacPherson et al., IRL Press at Oxford University Press 1991). However, PCR conditions used for each application reaction are empirically determined. A number of parameters influence the success of a reaction. Among them are annealing temperature and time, extension time, Mg^{2+} ATP concentration, pH, and the relative concentration of primers, templates, and deoxyribonucleotides. Provided herein are conditions that are optimized for replicating the human I_{1B}-AR and ϑ_2-AR gene with the aforementioned primer pairs. Preferably, the template nucleic acids are denatured by heating to at least about 90°C prior to the polymerase reaction. Typically, approximately 30 cycles of amplification are executed using denaturation at a range of 90°C to 95°C for 0.5 to 1 minute, annealing at a temperature ranging from 55°C to 65°C for 1 to 2 minutes, and extension at 68°C to 75°C for at least 1 minute with the final cycle extended to 10 minutes. Each PCR reaction typically contains 100 ng template nucleic acids, 1 TM of upstream and downstream primers, and 0.1-0.2 mM dNTP of each kind, and 1 to 5 units of commercially available DNA polymerases.

After amplification, the resulting DNA fragments can be detected by agarose gel electrophoresis followed by visualization with ethidium bromide

staining and ultraviolet illumination. A specific amplification of the receptor gene can be verified by demonstrating that the amplified DNA fragment has the predicted size, exhibits the predicated restriction digestion pattern, and/or hybridizes to the correct cloned DNA sequence.

Detection of genetic variations in the human I_1B-AR or ϑ_2-AR

Whereas there is a clear association between receptor dysfunction and various disorders, the search for the genetic basis for abnormalities of I_{1B}-AR or ϑ_2-AR has been hampered by the continued lack of a rapid, informative and cost-effective methods applicable for analyzing a large volume of clinical samples. Without a large-scale screen of I_{1B}-AR and ϑ_2-AR genes obtained from normal and diseased individuals, it is difficult to establish the clinical significance of any detected alterations in these receptors.

Accordingly, this invention provides a method for a rapid detection of genetic variations in I_{1B}-adrenergic receptor of a subject, and also a method of detecting genetic variations in ϑ_2-AR. Each method generally comprises the following steps: a) providing a biological sample containing nucleic acid molecules from the subject; b) amplifying a segment of the gene by a primer pair as described above; and c) identifying sequence variations of the resulting amplified products relative to a control, e.g., by employing one or more of the following analytical steps: nucleotide sequencing, single-strand conformation polymorphism assay, allele-specific oligonucleotide hybridization, Southern blot analysis, and restriction endonuclease digestion.

In the method of the present invention, the test nucleic acid can be obtained from any of a variety of sources. For example, the test nucleic acid can be prepared from cells within a body fluid of the subject or from cells constituting a body tissue of the subject.

The particular body fluid from which the cells are obtained can be selected from the group consisting of blood, pleural fluid, ascites, urine and spinal fluid.

Furthermore, the particular body tissue from which the cells are obtained can be selected from the group consisting of skin, endometrial, intrauterine, oral mucosa and cervical tissue. Both normal and tumor tissues can be used.

When assaying for genetic variations of I_{1B}-AR or ϑ_2-AR, the test nucleic acid can be, e.g. genomic DNA, cDNA or mRNA, depending on the type of sequence variants that is to be determined. To identify alternatively spliced variants, mRNA should be used. However, when examining polymorphic alleles of the receptor genes in different individuals, the test nucleic acid is preferably genomic DNA. When detecting point mutations, the preferred test nucleic acid is also genomic DNA. Genomic DNA can be prepared, e.g. as described by Sambrook et al. ("Molecular Cloning: A Laboratory Manual", Second Edition, 1989) or using commercial kits following the manufacturers' instructions. cDNA and mRNA can be isolated according to standard techniques in the art.

Amplification of the nucleic acids encoding receptor genes can be carried out by any means employing a primer-dependent polymerase known in the art. Such polymerase includes natural or recombinant DNA-polymerases. A preferred amplification method is PCR. In addition to the benefits of simplicity and efficiency, PCR using the primer sets provided herein generates a large amount of gene segments that can be directly used for subsequent DNA analyses without further genetic manipulation of the amplified products.

A variety of analytical techniques are available in the art for detecting or confirming sequence variations in a polynucleotide. The choice of a particular technique depends on the nature of the variations. Restriction endonuclease digestion can be employed to detect a sequence variation that creates or abolishes a restriction enzyme recognition site. Single-strand conformation polymorphism assay can be used for the detection of single base substitutions. Oria et al., *Proc. Natl. Acad. Sci. USA* **86**:2766-2770 (1989); Gaudamo et al., *Proc. Natl, Acad. Sci. USA* **88**: 5413-5417. This method is based on the observation that changes in the nucleotide sequence affect single-strand conformation, and thus resulting in altered electrophoretic mobility. In addition, allele-specific oligonucleotides can

be employed in a hybridization procedure to differentiate polymorphic alleles that carry changes in single nucleotide positions. This procedure can be performed when the sequence variations are known. Furthermore, Southern blot analysis can be used alone or in conjunction with the above-described methods to detect changes in hybridization pattern with a gene specific probe.

A preferred method of detecting genetic variations in I_{1B}-AR and ϑ_2-AR gene is by sequencing the amplified products. Since the location and nature of the alterations are often unknown, and most of the aberrant I_{1B}-ARs and ϑ_2-ARs identified thus far carry changes in single amino acid residues, it is desirable to examine the nucleotide sequence of the amplified products. Distinguished from the previously reported sequencing analyses involving nested primers (C.S. Ramarao et al., *J. Biol. Chem.* **267**:21936-21945, 1992; J. Turki et al., *J. Clin. Invest.* **95**:1635-1641, 1995), sequencing the amplified products of the present invention is performed using the same primer employed in the amplification reaction (see Example 5). Nested primers are generally required for obtaining an accurate reading of the nucleotide sequence because of a frequently-encountered problem, namely product heterogeneity as a result of mispriming or misextension of the amplification primers. The method of this invention does not suffer from the problems associated with nonspecific amplification. Primers of the present invention amplify specific gene segments, and provide reliable and reproducible reading of the nucleotide sequences. DNA sequencing is preferably carried out by an automated sequencer (e.g. Applied Biosystems, model 377), which reads at least 400 bases per reaction. DNA sequences can also be determined by methods employing gene chip technology, such as that offered by Affymetrix. Furthermore, sequencing can be performed manually using commercial kits (e.g. UBI) according to manufacturer's instructions.

In carrying out the aforementioned DNA analyses for determining sequence variations of the receptor genes in a subject or in a population of interest, one generally includes appropriate controls for a comparison. It is often preferable to conduct the analyses on the test sample and on the control samples

in parallel. Since some mutations and polymorphisms occur more frequently than others, a common frame of reference is preferably the receptor gene as that reported in the original cloning (C.S. Ramarao et al., *J. Biol. Chem.* **267**:21936-21945, 1992; B.K. Kobilka et al. *Proc. Natl. Acad. Sci. USA* **84**:46-50, 1987). To determine whether the polymorphisms and/or mutations detected in the test subject are homozygous or heterozygous, it is preferable to include controls whose genotypes with respect to those variations have been previously determined. Further details and alternatives for control samples are provided in the descriptions that follow.

Diagnosing diseases that are associated with genetic alterations of human I_{1B}-AR or ϑ_2-AR

It has been known for almost 50 years that there is an association between dysfunctions of I_{1B}-AR and ϑ_2-AR and various disorders. In recent years, a wealth of evidence has emerged that implicates a causal role of certain mutations and polymorphisms of these receptor genes in certain types of asthma (J. Turki et al., *J. Clin. Invest.* **95**:1635-1641, 1995), hypertension (L.P.Svetkey et al., *Hypertension* **27**: 1210-1215, 1996) and other cardiovascular disorders. Ohe et al. reported that Ban-I RFLP of ϑ_2-AR gene is associated with bronchial asthma (M. Ohe et al. *Thorax* **50**: 353-359, 1995). Liggett et al. have shown that the polymorphic variant encoding glycine instead of arginine at position 16 is overrepresented in patients with nocturnal asthma (S.B. Liggett et al., *Clinical and Experimental Allergy* **25(2)**: 89-94, 1995). Subsequent studies involving site-directed mutagenesis and recombinant expression revealed that the Glycine 16 variant exhibits an augmented receptor downregulation, which is consistent with the nocturnal downregulation of ϑ_2-AR and the attenuated response to agonists observed in nocturnal asthma. More recently, the same polymorphic variant was found to be associated with hypertension. The altered receptor is likely to be responsible for the attenuated vasodilation and increase in blood pressure

commonly found in hypertensives (P. Kontanko et al., *Hypertension* **30(4)**: 773-776, 1997).

An involvement of I-ARs in hypertension has also been noted. In fact, I-AR antagonists are widely used as anti-hypertensive drugs. Recent studies with I_{1B}-AR-deficient transgenic mice have demonstrated that within the I-AR family, I_{1B}-AR in particular, mediates agonist-induced blood pressure and vascular contractile responses (A. Cavalli et al. P*roc. Natl. Acad. Sci.* USA, **94**: 11589-11594, 1997). Therefore, it is highly likely that genetic alterations of I_{1B}-AR leading to enhanced vasoconstrictions, and/or genetic alterations of ϑ_2-AR resulting in attenuated vasodilation, contribute to human hypertension.

Accordingly, this invention provides a simple and rapid method for diagnosing diseases associated with genetic alterations of I_{1B}-adrenergic receptors. This invention also provides a method for diagnosing diseases associated with genetic alterations of β_2-adrenergic receptors. Each method generally comprises the following steps: a) providing a biological sample of the subject containing nucleic acid molecules; b) amplifying a segment of the gene by employing a pair of DNA primer described herein, a primer-dependent DNA polymerase, and a sufficient amount of deoxyribonucleotides to generate a plurality of amplified segments of the gene; c) screening the resulting segments for genetic variations relative to a control involving at least one analytical step as described herein; and d) determining a correlation of the detected variations between the subject and a control.

For the purpose of this invention, a nucleic acid-containing sample is any sample containing nucleic acid suitable for use in an amplification procedure. As used herein, amplification means any method for replicating a nucleic acid with the use of a primer-dependent polymerase. In a preferred embodiment, the amplification is effected by means of PCR using two sets of I_{1B}-AR or ϑ_2-AR gene specific primers as described above. Amplified products are subjected to one or more of the following analyses, including nucleotide sequencing, single-strand conformation polymorphism assay, allele-specific oligonucleotide

hybridization, Southern blot analysis, and restriction endonuclease digestion. A preferred analysis is direct nucleotide sequencing.

In diagnosing diseases associated with genetic alterations in β_{1B}-AR or β_2-AR, one typically conducts a comparative analysis of the subject and appropriate controls. Preferably, a diagnostic test includes a control sample derived from a subject (hereinafter positive control), that carries the same variant receptor gene and exhibits syndromes that are characteristic of the disease of interest. More preferably, a diagnosis also includes a control sample derived from a subject (hereinafter negative control), that retains the "wild-type receptor gene" (having the nucleic acid sequence as that reported in the original cloning) and lacks the clinical syndromes. A positive correlation between the subject and the positive control with respect to the identified alterations indicates the presence or a predisposition of said disease. A lack of correlation between the subject and the negative control confirms the diagnosis.

In a preferred embodiment, the method is used for diagnosing nocturnal asthma based on glycine 16 polymorphism in β_2-AR gene. In another embodiment, the method is used for a diagnosis of essential hypertension based on the same genetic polymorphism. While assaying for glycine 16 polymorphism, genomic DNA of the test subject can be obtained from a blood sample. The N-terminal fragment A (as shown in Figure 2) that encompasses sequences encoding the residue glycine 16 can be amplified using primers SEQ ID NOS:5 and 6, or derivatives thereof. Direct sequencing of the amplified products using the same primers employed in the amplification procedure may be performed to detect the single base change (adenosine to guanosine) at position 46 (numbering from the start codon) that results in amino acid substitution of arginine with glycine. Homozygosity of glycine 16 indicates the presence or a predisposition to nocturnal asthma and/or essential hypertension. Glycine 16 homozygotes will be apparent by comparing the sequence peaks from the subject sample to those of the controls (for an example of the sequence output from an automated DNA sequencer, see Figure 5 or 6). A single peak corresponding to

nucleotide guanosine at position 46 is indicative of glycine 16 homozygosity. Two overlapping peaks, each representing guanosine or adenosine respectively at position 46, suggest heterozygosity of glycine 16. Finally, a single peak representing adenosine shows that the subject is homozygous in arginine 16.

The following Examples are provided to further assist those of ordinary skill in the art. Such examples are intended to be illustrative and therefore should not be regarded as limiting the invention. A number of exemplary modifications and variations are described in this application and others will become apparent to those of skill in this art. Such variations are considered to fall within the scope of the invention as described and claimed herein.

EXAMPLES

Example 1

Generation of α_{1B}-AR gene specific primer sets

As an illustration of the primer sets for use in the present invention, we have designed two sets of primers (having the properties described above) that amplify two overlapping fragments encompassing the entire exon 1 sequence of the human α_{1B}-AR gene. A first exemplary set contains two opposing primers each having the nucleotide sequence 5'-CGGGGGAAGCAAAGTTTCA-3' (SEQ ID NO:1) or
5'-CGGCAGTACATGACTAGAAT-3' (SEQ ID NO:2). A second exemplary set consists of an upstream and downstream primer having the nucleotide sequences
5'-CTCTCCTTGGGTGGA AGGA-3' (SEQ ID NO:3) and
5'-AGCTCATCAGTAAACCCAAG-3' (SEQ ID NO:4), respectively. Individual primers of each set are (a) non-self and non-cross hybridizing, (b) having a preferred length of 18 to 20 nucleotides, and (c) a comparable melting temperature in the range of 54 to 60 ^{0}C. The first primer set amplifies the N-

terminal half of exon 1 (fragment A in Figure 1), and yields a population of substantially homogeneous products as evidenced by a single DNA band of predicted size on an electrophoretic agarose gel (Lane 1, Figure 4). The second primer set amplifies the C-terminal half of exon 1 (fragment B in Figure 1), generating a discrete fragment of 473 base pairs as shown in Lane 2 of Figure 4. The resulting products were substantially homogenous, and thus suitable for direct sequencing analysis (see Example 5). In addition, primers of these sets, namely primer SEQ ID NOS:1, 2 and 3 are capable of extending their 3' end sequences complementary to a template in a DNA polymerase reaction, such as a sequencing reaction. An exemplary sequence output of fragment A generated by an automated sequencer using primer SEQ ID NO:1 is depicted in Figure 5.

Example 2

Generation of β_2-AR gene specific primer sets

Exemplary primer sets specific for β_2-AR gene were also designed to exhibit each of the characteristics listed above. Exemplary set 1 contains two primers each having a nucleotide sequence 5'-GAATGAGGCTTCCAGGCGTC-3' (SEQ ID NO:5) or 5'-GATGATGCCTAACGTCTTG-3' (SEQ ID NO:6). Exemplary set 2 consists of two primers having the nucleotide sequences 5'-TTCTACGTGCCCCTG GTG-3' (SEQ ID NO:7) and 5'-TCCTCTAGGACTAAAGCTC-3' (SEQ ID NO:8), respectively. Whereas the first set amplifies the N-terminal half of the coding sequences as shown in region A of Figure 2, resulting in a large fragment of 917 nucleotide bases; the second set amplifies the C-terminal half of the coding sequences, yielding a fragment of 862 base pairs. The products generated by these primer sets are of substantial homogeneity as reflected by discrete bands of predicted sizes on an electrophoretic agarose gel (Lanes 3 and 4 in Figure 4). Furthermore, at least one primer of each set, namely primer SEQ ID NOS:5, 6 and 7, can be used for direct sequencing of the amplified fragments. An exemplary sequence profile of

fragment A, which was determined by an automated sequencer using primer SEQ ID NO:6 is shown in Figure 6.

Example 3

Use of α_{1B}-AR primer sets to amplify α_{1B}-AR coding sequence from human genomic DNA

Approximately 3ml of heparinized human blood was drawn from healthy volunteers. Genomic DNA was extracted using a commercial DNA Isolation Kit (Gentra System, Inc.) according to accompanying instructions. α_{1B}-AR primer sets 1 and 2 (see Table 1) were used to amplify the coding sequence of exon 1 by PCR.

PCR was performed in a 100 Tl reaction mixture containing 100ng of purified genomic DNA, 1 micromole of upstream and downstream primers, 2.5 mM $MgCl_2$, 50 mM NaCl, 10 mM Tris-Cl, pH 8.3, 0.2 mM each dNTP (Pharmacia), and 5 units of Amplitaq Gold Polymerase (Perkin Elmer). The reaction mixture was initially incubated at 95 ^{0}C for 10 minutes and then subjected to 35 cycles of PCR in a Perkin-Elmer/Cetus thermocycler as follows:

denaturation 94°C, 30 seconds

annealing 60°C, 1.5 minutes

extension 72°C, 1.5 minutes - final extension 10 minutes.

After amplification, the products from each amplification reaction were resolved by electrophoresis on a 1% Seakem agarose gel, and then visualized upon staining with ethidium bromide. As shown in lanes 1 and 2 of Figure 4, each primer set generated a discrete DNA band of predicted size.

Example 4

Use of β_2-AR primer sets to amplify β_2-AR coding sequence from human genomic DNA

Human genomic DNA was prepared from a blood sample as described above. Two sets of β_2-AR primers were used to amplify two overlapping fragments that encompass the entire coding sequence of β_2-AR gene (see Table 1). Amplification was performed by PCR under the same conditions as described in Example 3. The resultant products were separated on an agarose gel. As indicated in lanes 3 and 4 of Figure 4, the two primer sets generated discrete fragments of approximately 917 base pairs and 862 base pairs, respectively.

Example 5

Sequence Analysis

The PCR products of expected sizes were cut from the gel and the DNA was purified using QIAquick Gel Extraction Kit. The extracted DNA was resuspended in Tris-EDTA buffer (10mM Tris-Cl, 1mM EDTA, pH 8.0) and concentrated using a Centricon Concentrator (Amicon). The purified gene fragments were then sequenced by an automated DNA sequencer (Applied Biosystems, model 377) using one or more of the same primers employed in PCR. The upstream primer SEQ ID NO:1 and the downstream primer SEQ ID NO:6 were used for sequencing the amplified products, fragment A of the human I_{1B}-adrenergic receptor gene and fragment A of β_2-adrenergic receptor gene, respectively. As shown in Figure 5 and 6, each sequencing read approximately 550 bases. Other primers described herein including primer SEQ ID NOS:1, 2, 3, 5, and 7 can also be used for direct sequencing with high reliability. By use of an automated sequencer and sequencing PCR products from in excess of 15 different subjects, we obtained consistent results in accordance with the published coding sequences of the human β_2- and exon 1 of the human I_{1B}-adrenergic receptor.

Repeated sequencing of PCR products of the same individuals revealed a 100% reliability of our PCR methods without requirement for repeat isolation of PCR fragments. Occasionally occurring "ambiguous reads", which are the result of a reading error of the automated sequencer, can generally be corrected afterwards without re-isolating and sequencing the PCR fragments (Figures 5 and 6).

Example 6

Use of α_{1B}-AR or β_2-AR primer sets for diagnosing diseases associated with genetic alterations in α_{1B}-AR and β_2-AR

α_{1B}-AR or β_2-AR primer sets are used to amplify large fragments of the receptor genes, which can subsequently be employed in sequence analyses to detect, prognose, diagnose, or monitor various disease conditions based on sequence variations identified in the amplified fragments. Disease conditions resulting from genetic alterations of α_{1B}-AR and β_2-AR can include pulmonary, endocrine-metabolic, neuronal, and cardiovascular disorders. For example, the α_{1B}-AR or β_2-AR gene of subject X is amplified using the primer sets disclosed herein, by a method such as PCR. The resultant gene fragments are analyzed by at least one of the sequence analytical steps, preferably by e.g. direct nucleotide sequencing. By comparing the nucleotide sequence of the receptor gene of subject X to the wild-type receptor sequence, the occurrence of a mutation and/or polymorphism is determined. Further sequence comparison with a positive control, that carries the same variant receptor gene and exhibits syndromes characteristic of the disease of interest, establishes a positive correlation between the two, which indicates the presence or a predisposition of said disease in subject X. Additional sequence comparison with a negative control that retains the wild-type receptor gene and lacks the clinical syndromes confirms the diagnosis.

Table 1.

I_{1B}-adrenergic receptor		Primer-sequence
Primer set no. 1	Upstream (SEQ ID NO:1)	5'-CGG GGG AAG CAA AGT TTC A-3'
	Downstream (SEQ ID NO:2)	5'-CGG CAG TAC ATG ACT AGA AT-3'
Primer set no. 2	Upstream (SEQ ID NO:3)	5'-CTC TCC TTG GGT GGA AGG A-3'
	Downstream (SEQ ID NO:4)	5'-AGC TCA TCA GTA AAC CCA AG-3'
9_2-adrenergic receptor		Primer-Sequence
Primer set no. 1	Upstream (SEQ ID NO:5)	5'-GAA TGA GGC TTC CAG GCG TC-3'
	Downstream (SEQ ID NO:6)	5'-GAT GAT GCC TAA CGT CTT G-3'
Primer set no. 2	Upstream (SEQ ID NO:7)	5'-TTC TAC GTG CCC CTG GTG-3'
	Downstream (SEQ ID NO:8)	5'-TCC TCT AGG ACT AAA GCT C-3'